

## **FoxO is required for the activation of hypertrehalosemic hormone expression in cockroaches**

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## ABSTRACT

**Background:** FoxO proteins are a subgroup of the Forkhead-box family of transcription factors, which function as the main transcriptional effectors of the Insulin Receptor pathway. This pathway, activated by the binding of insulin or IGFs (or insect insulin-like peptides), promotes the phosphorylation and inactivation of FoxO because of its export from the nucleus to the cytoplasm. The homolog of FoxO in the cockroach *Blattella germanica* works in a situation of nutrient shortage by inhibiting the endocrine induction of reproduction.

**Methods:** Using *Blattella germanica* as a model, we studied the functions of FoxO using RNA interference methodologies. We analyzed the mRNA levels of hypertrehalosemic hormone (HTH) and genes related to lipolysis, glycogenolysis and gluconeogenesis and quantified triacylglycerides, glycogen and trehalose.

**Results:** FoxO knockdown eliminates the starvation-induced expression of HTH in the corpora cardiaca. In addition, FoxO knockdown prevents the activation of the expression of Brummer lipase, glycogen phosphorylase and phosphoenolpyruvate carboxylase in the fat body of starved females.

**Conclusions:** Starvation-induced activation of FoxO stimulates the transcription of different genes related to catabolic processes, including HTH and genes involved in lipolysis, glycogenolysis and gluconeogenesis.

**General significance:** Our results show conservation in the action of the transcription factor FoxO in the activation of catabolic processes from basal insects to vertebrates. The results also describe a new and essentially different mode of action of transcription factor FoxO, which works through the activation of neuropeptide HTH expression, which will subsequently produce its own catabolic stimulatory function.

**Keywords:** FoxO, insulin, catabolism, hypertrehalosemic/adipokinetic hormone, glucagon, *Blattella germanica*

## 1. Introduction

Multicellular organisms have a complex system to maintain homeostasis and to regulate their response to changing nutrient conditions. Hormone-controlled anabolic and catabolic processes serve to regulate the flow of carbon and ensure adequate levels of circulatory sugars. One difference between insects and mammals, among other major distinctions, is that the primary circulating sugar in insects is trehalose, the non-reducing disaccharide of glucose. Insulin signaling is involved, however, in the regulation of circulating sugar levels in both cases [1-3]. Insulin-like peptides in insects are homologous to mammal insulin as well as being functionally equivalent [4]. Insect genomes contain multiple genes encoding insulin-like molecules: eight in *Drosophila melanogaster* [5-7], seven in the mosquito *Anopheles gambiae* [8], and as much as thirty-nine in the silkworm *Bombyx mori* [9-10].

The main insect hormones that counteract insulin signaling at metabolic levels are the adipokinetic/hypertrehalosemic hormones (AKH/HTH) which are the insect functional analogs of glucagon [11-12]. This group comprises a large family of peptides synthesized in the corpora cardiaca (CC), a gland placed in a retrocerebral position and innervated to the brain [13]. Depending on the species, the activity of these peptides has been described as hypertrehalosemic, hyperlipemic (adipokinetic), hyperprolinemic or as a mixture of these activities [14]. Thus, in cockroaches, peptide hormones belonging to this family have been described as hypertrehalosemic since their discovery [15] and were named accordingly. In the cockroach *Blattella germanica* a hypertrehalosemic hormone (HTH) has already been described and its function in the induction of trehalose release demonstrated [12].

Besides the effect of anabolic and catabolic hormones, the signaling pathways that regulate levels of circulatory trehalose and glucose are also affected by transcription factors, for example, the Forkhead-box O (FoxO). To date, four FoxO genes have been described in the mouse genome [16], although in most invertebrate species only one gene has been detected, e.g. DAF-16 in *Caenorhabditis elegans* [17] and dFoxO in *D. melanogaster* [18]. FoxO is the main transcriptional effector of the Insulin Receptor pathway. When the pathway is active, mainly in a nutrient-dependent way,

FoxO is phosphorylated and retained in the cytoplasm, whereas the inactivation of the pathway allows dephosphorylated FoxO to enter into the nucleus and exert its transcriptional activity [19-20]. Kramer and co-workers [21] demonstrated that FoxO transcription factor is activated upon amino acid starvation in *D. melanogaster* and that this activation is required for the animals to survive under this adverse condition. Genome-wide evolutionary conservation of FoxO targets between the fly and the worm *C. elegans* have been reported [22]. The regulation of metabolism related genes via FoxO has been also described in mammals [23], which confirms a general action of FoxO transcription factors on metabolism regulation. In particular, the transcriptional activity of FoxO promotes catabolic routes and inhibits anabolic ones [20, 23-24].

In *B. germanica*, one cDNA coding for a FoxO homolog (BgFoxO) has recently been cloned, and its inhibitory role on juvenile hormone biosynthesis and vitellogenin production in a situation of nutrient shortage has been reported [25]. In the present paper, we use *B. germanica* as a model for analyzing the function of FoxO on the activation of catabolic pathways in insects. We have studied the effect of FoxO knockdown, using it to analyze FoxO function in the regulation of the endocrine and metabolic changes that occur during insect starvation.

Two different (and in principle unrelated) systems control energy expenditure in insects [26]. One of them relies on the hormonal signal of the neuropeptide AKH/HTH which, after binding to a G protein-coupled receptor, produces an activation of protein kinase A (PKA) and an increase of the corresponding intracellular second messengers, calcium and cyclic AMP, which in turn activates some catabolic enzymes, among them glycogen phosphorylase (GlyP) [14, 26]. An analogous system functions in vertebrates, where stimulation of glucagon and  $\beta$ -adrenergic receptors activates PKA and a series of energy mobilization enzymes [27]. The second system that controls energy expenditure in insects works via the regulation of Brummer lipase (Bmm) activity. This enzyme, homologous to the mammalian adipose triglyceride lipase (ATGL), promotes the mobilization of lipids from the fat body in response to food deprivation [28]. Recent studies have

demonstrated that in *D. melanogaster*, Bmm expression is activated through a direct binding of the transcription factor dFoxO to its promoter [29].

Results presented in this work indicate that FoxO activates the expression of HTH in the CC of starved animals. In addition, FoxO activates the expression of critical enzymes in lipolysis, glycogenolysis and gluconeogenesis. Together our results show that FoxO is a key factor in the activation of catabolism in insects, through both the activation of an endocrine pathway (HTH) and through the activation of enzyme expression.

## **2. Material and methods**

### *2.1. Insects*

Specimens of *B. germanica* were obtained from a colony reared with dog chow and water, in the dark at  $30 \pm 1^\circ\text{C}$  and 60-70% relative humidity. For the study of gene expression levels during the first gonadotrophic cycle, virgin females were used. For the RNAi experiments, we induced a second gonadotrophic cycle by removing the ootheca at the twelfth day of its transport period. For the starvation experiments, animals received only water after the imaginal moult or after the induction of the second gonadotrophic cycle. Dissections of the different tissues were carried out on carbon dioxide-anesthetized specimens. After dissection, tissues were immediately frozen in liquid and stored at  $-80^\circ\text{C}$

### *2.2. Cloning of Bmm, GlyP, AspAT, PC and PEPCK cDNAs*

Degenerated primers based on conserved regions of insect brummer lipase (Bmm), glycogen phosphorylase (GlyP), aspartate aminotransferase (AspAT), pyruvate carboxylase (PC) and phosphoenol pyruvate carboxykinase (PEPCK) sequences were used to obtain a *B. germanica* homologue cDNA fragment of each of these genes. Primers can be found as Supplementary data, Table 1. The amplified fragments were subcloned into the pSTBlue-1 vector (Novagen; 70596) and

sequenced. In the case of PEPCK, 3'- and 5'-RACE methodologies (5'- and 3'-RACE System Version 2.0; Invitrogen) were used to complete the sequence.

### 2.3. RNA extraction, cDNA synthesis and real-time PCR analyses

The CC, fat body and muscle expression levels of the studied genes were analyzed using real-time PCR. cDNA was synthesized from total RNA as previously described [25, 30]. An amount of 0.5 mg of total RNA was used in the case of fat bodies, whereas in the case of muscle and CC, the total amount of RNA obtained from all legs and the whole gland, respectively were used. The absence of genomic contamination was checked using a control without reverse transcription. cDNA levels were quantified by using iQSYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad) as previously described [31]. Primer sequences to amplify BgFoxO and BgActin 5C (used as housekeeping gene) can be found in [25], whereas sequences to amplify BgHTH, BgBmm, BgGlyP, BgAspAT, BgPC, BgPEPCK can be found as Supplementary data, Table 1. All reactions were run in duplicate or triplicate. Real-time data were collected by iQ5 optical system software v.2.0 (BioRad). Results are given as copies of a determined mRNA per copies of BgActin 5C.

### 2.4. RNA Interference

Systemic RNAi in vivo in females of *B. germanica* was performed as previously described [25, 30]. A 298 bp fragment (dsFoxO) encompassing the protein C-terminus and part of the 3' non-coding region, spanning position 1918 to 2215 of the BgFoxO cDNA, was used to generate dsRNA [25]. As a control, a heterologous 307 bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsMock) was used [32]. To be confident that the treatment produced a sufficient BgFoxO reduction, we performed long time treatments. They consist in the injection of 2 µg of the dsRNA fragment into the abdomen of females in the first day of ootheca transport. Twelve days later, we removed the ootheca, triggering the

beginning of a second gonadotrophic cycle in all respects similar to the first one. In addition, using this experimental model we avoid the possible developmental phenotypes caused by RNAi treatment at the nymphal stages and only concentrate in the effects of BgFoxO RNAi treatment in the adult. After removing the ootheca, one group of each treatment (dsMock or dsFoxO) was maintained with food and water (fed group), whereas another group was kept with water but without food (starved group). Dissections were performed immediately after ootheca removal, so before the separation of fed and starved groups (0d) and 5 days later (5d).

### *2.5. Triglyceride measurement and Nile Red staining*

For triglyceride measurement, fat body was homogenized in 100µl extraction buffer containing 200 mM potassium phosphate pH 4.8, 0.1 % Tween 20 (PBS/T) and immediately incubated for 5 min. at 70 °C. One aliquot (20 µl) of the extract was mixed with 20 µl triglyceride reagent (Sigma) containing lipase activity. A second aliquot was mixed with the same amount of PBS/T for calculating the levels of free glycerol in the sample. Both tubes were incubated at 37 °C for 30 min, and centrifuged at maximum velocity for 3 min at room temperature. One aliquot (30 µl) was incubated with 100 µl free glycerol reagent for 5 min at 37 °C. Absorbance was read at 540 nm. Glycerol levels were determined using a standard curve submitted to the same conditions. Free glycerol calculated from the sample without lipase treatment was subtracted from the glycerol values in the treated samples. Triglycerides levels were estimated from the amount of glycerol obtained after lipase treatment.

Fat body lipid droplets were visualized using Nile Red staining. Pieces of abdominal fat body were mounted in a medium containing 50% glycerol/PBS/T, Nile Red 1:55,000 (Molecular Probes). Tissues were analyzed using a Zeiss AxioImager.Z1 microscope (Carl Zeiss MicroImaging) for visualization of lipid droplets.

### *2.6. Glycogen and protein measurements*

Fat body was homogenized in 150  $\mu$ l extraction buffer containing 200 mM sodium acetate pH 4.8. The homogenates were centrifuged at 10,000xg for 10 min at 4 °C. One aliquot (50  $\mu$ l) of the supernatant was mixed with 1 unit  $\alpha$ -amyloglucosidase (Sigma). A second aliquot was mixed with the same amount of buffer for calculating the levels of free glucose in the sample. Both tubes were incubated at 40 °C for 4h. Samples were then incubated with glucose oxidase reagent for 30 min at 37 °C, followed by the addition of 100  $\mu$ l of 12N H<sub>2</sub>SO<sub>4</sub>. The absorbance of each sample was read at 540 nm. Glucose content was quantified according to the manufacturer's instructions (Glucose Assay (GO) Kit; Sigma). Glycogen content was determined using a glucose standard curve submitted to the same conditions. Free glucose calculated from the sample without  $\alpha$ -amyloglucosidase treatment was subtracted from the glycogen values.

The protein content of samples was determined according to Bradford's method, using bovine serum albumin as standard.

## *2.7. Haemolymph collection and trehalose measurement*

Haemolymph samples (5 $\mu$ l) were collected in a microcapillary pipette, after cutting off the metathoracic legs, diluted in 5 $\mu$ l NaCl 0.4 M plus 4 % Protease Inhibitor (Sigma), fast frozen in liquid nitrogen and stored at -80°C. Samples were centrifuged at 10,000xg for 10 min at 4 °C and 200  $\mu$ l 0.25 M Na<sub>2</sub>CO<sub>3</sub> buffer were added to the supernatants. Samples were mixed by vortexing them for 1 min and incubated at 96 °C for 2 h to inactivate all enzymes. Then, 120  $\mu$ l of 1 M acetic acid and 480  $\mu$ l 0.25 M Na-acetate (pH 5.2) were added, and the solution was centrifuged at 12,000 rpm, room temperature for 10 min. One aliquot (100  $\mu$ l) of the supernatant was incubated overnight at 37 °C with 2  $\mu$ l porcine kidney trehalase (Sigma) in order to convert trehalose into glucose. A second aliquot was mixed with the same amount of buffer and incubated in the same way for calculating the levels of free glucose in the sample. The amount of glucose in 50  $\mu$ l of the above solutions was calculated as in the case of glycogen measurement but using a trehalase-treated trehalose standard



curve. Trehalose concentration was determined after subtracting the amount of glucose without trehalase treatment.

### 3. Results

#### 3.1. Cloning of *Bmm*, *GlyP*, *AspAT*, *PC* and *PEPCK* cDNAs

Using degenerate primers and cDNA from diverse tissues, we attained fragments of differing lengths for the different studied genes . In the case of PEPCK, we employed 5'- and 3'-RACE methods to attain the full-length sequence. A BLAST database search indicated that the proteins were the *B. germanica* orthologs of Brummer lipase (BgBmm, GenBank accession number: HG005308), glycogen phosphorylase (BgGlyP, HG005309), aspartate aminotransferase (BgAspAT, HG005310), pyruvate carboxylase (BgPC, HG005311) and phosphoenolpyruvate carboxykinase (BgPEPCK, HG005312).

#### 3.2. *BgFoxO* knockdown prevents the starvation-induced activation of *BgHTH* expression

mRNA levels of BgHTH were analyzed in the CC of fed and starved, 5 day old adult females. Results showed that BgHTH expression is upregulated in starved females (Fig. 1A).

To study the effect of BgFoxO in the CC, its expression was depleted by RNAi. dsRNA targeting BgFoxO (dsFoxO) or a non-homologous dsRNA (dsMock) were administrated in the first day of ootheca transport. The ootheca was removed 12 days later and a second gonadotrophic cycle was triggered. A group of females were normally fed, whereas a second group was maintained without food and only water was provided. Dissections were performed the day of the ootheca removal and 5 days later. Results showed that dsFoxO treatment induced significant decreases of mRNA BgFoxO in the CC of all of the experimental conditions studied (0 d, 5 d fed and 5 d starved). In particular, in the starved females, dsFoxO treatment produced an 82%

significant reduction in BgFoxO mRNA levels. BgHTH mRNA levels were also studied under the experimental conditions. Results showed that, although starvation induced an increase of BgHTH mRNA levels the increase was not observed in starved dsFoxO females (Fig. 1A, C).

### *3.3. Lipolysis is reduced and TAG are accumulated in BgFoxO-depleted females*

One of the enzymes involved in fat body lipolysis is Bmm. After proving the efficiency of dsFoxO treatment on the reduction of BgFoxO mRNA in fat body (Fig. 2A), BgBmm mRNA levels were analyzed for the different experimental conditions. Results showed that BgFoxO knockdown produced a decrease in BgBmm levels in 0 and 5 day old fed females (Fig. 2B). Results also indicated that starvation induced a 2.3-fold significant increase in BgBmm mRNA in control females ( $p = 0.0022$ , Student's  $t$  test), whereas dsFoxO treatment eliminated this starvation-induced increase in BgBmm mRNA levels (Fig. 2B).

In parallel to BgBmm expression levels, TAG content in abdominal fat body was higher in dsFoxO-treated females (Fig. 2C, D). Even during starvation, when control (dsMock) females had exhausted the whole of their TAG stores, dsFoxO starved females maintained TAG levels that were more than 20-fold higher (Fig. 2C).

### *3.3. Glycogenolysis is reduced in fat body but not in muscle in BgFoxO-depleted females*

Glycogen phosphorylase (GlyP) is the key enzyme in glycogenolysis. We measured BgGlyP mRNA and glycogen levels in fat body and muscle of females under the different experimental conditions. Fat body BgGlyP mRNA levels underwent a six-fold significant increase ( $p < 0.0001$ , Student's  $t$  test) in starved females compared to fed females, but this increase was reduced in the case of dsFoxO-treated starved females (Fig. 3A). In parallel, fat body glycogen levels were lower in 0 and 5 day old, fed dsFoxO females compared to the controls. In the case of

starved females, the expected very low fat body glycogen levels observed in control animals was significantly increased in dsFoxO (Fig. 3B).

In the case of muscle, after confirming that dsFoxO also triggered a reduction of BgFoxO mRNA in muscle tissue (Fig. 4A), we did not observe a reduction of BgGlyP mRNA in any of the cases, not even in starvation conditions (Fig. 4B). And, although we again found a reduction in glycogen levels in 0 and 5 day old fed females, we did not observe the increase in glycogen levels in the muscle of dsFoxO starved females which was observed in fat body for such case (Fig. 4C).

#### *3.4. BgFoxO knockdown reduces circulating trehalose levels and prevents the starvation-induced activation of BgPEPCK expression*

We also studied haemolymph trehalose concentration and mRNA levels of key enzymes involved in gluconeogenesis. Results showed that BgFoxO RNAi treatment produced a significant decrease in haemolymph trehalose in all the experimental conditions (Fig. 5).

With respect to gluconeogenesis, we analyzed mRNA levels of different enzymes involved in the process. A 7-fold significant increase in phosphoenolpyruvate carboxykinase (BgPEPCK) mRNA levels was observed in control starved compared to fed females ( $p < 0.0001$ , Student's  $t$  test). This increase was abolished by dsFoxO treatment (Fig. 6A). In addition, dsFoxO 5 day old fed females showed a significant reduction in fat body aspartate aminotransferase (BgAspAT) mRNA levels compared to controls. A relative (27%) increase in BgAspAT levels was observed in control starved compared to fed females; this increase being eliminated in dsFoxO starved females, although the difference compared to controls was not significant (Fig. 6B). We didn't find any differences in pyruvate carboxylase (BgPC) mRNA levels between 5 day old control and dsFoxO, neither in the fed nor in the starved groups (Fig. 6C).

Muscle protein levels, estimated from the calculation of total protein in the legs, were constant across all the experimental conditions, except in the case of dsFoxO

starved females where a significant decrease, ca. 44%, in total protein was observed (Fig. 6D).

#### 4. Discussion

Energy homeostasis implies close regulation of the accumulation and mobilization of energy reserves. In different animal models, which include insects and mammals, these reserves are mainly glycogen and TAG [26, 33]. It has been described for insects that the control of energy expenditure is produced through two different systems. The first one starts with the binding of the neuropeptide AKH/HTH to its receptor and the subsequent activation of PKA which, in turn, induces glycogenolysis by activating GlyP at a post-translational level [14, 26]. The second system involves the stimulation of lipolysis through the activation of the expression of the lipase Bmm, mediated by the transcription factor FoxO [26, 28-29].

FoxO is the major coordinator of the transcriptional response to nutrients in *D. melanogaster*, *C. elegans* or mammals [20, 23, 34]. In the fruit fly, in fact, it has been estimated that 28% of the nutrient responsive genes are transcriptionally regulated by activated dFoxO [35]. Therefore, the study of the transcriptional changes that occur in a situation of FoxO knockdown are of great importance when studying metabolism in nutrient deprivation conditions.

In *B. germanica*, starvation induced the increase of HTH mRNA levels in the CC (Fig. 1A, C). An increase in HTH mRNA levels has also been reported in starved adult females of the cockroach *Blaberus discoidalis* [36]. The HTH mRNA increase observed in starved *B. germanica* was eliminated in BgFoxO knocked down (dsFoxO) females (Fig. 1B, C), which indicates that FoxO is required for the activation of HTH expression during starvation.

The fat body, the insect analog to vertebrate liver and adipose tissue, is the main energy reserve organ in insects. This energy is mainly stored in the form of glycogen and triacylglycerides (TAG). In *B. germanica*, although starvation induces an increase in fat body BgBmm mRNA levels, dsFoxO treatment reduces BgBmm mRNA and, concomitantly, increases TAG levels in both fed and starved females

(Fig. 2B-D). These results again indicate that BgFoxO activates BgBmm expression. As stated above, *D. melanogaster* Bmm is regulated through direct binding of dFoxO to its promoter [29], and in the tsetse fly, *Glossina morsitans*, FoxO knockdown also produces a decrease in Bmm mRNA levels [37].

In the mosquito *Culex pipiens*, short day lengths induce entering into diapause, characterized, among other features, by the sequestration of lipid reserves, whereas FoxO knockdown reduces lipid stores in diapausing females [38]. On the other hand, high abundance of FoxO protein has been detected in the fat body of *C. pipiens* females in early diapause, and these high levels of FoxO disappeared in response to juvenile hormone treatment [39]. These results point to a specific endocrine regulation and a different effect of FoxO in the animals that have an adult reproductive diapause.

GlyP is the enzyme responsible of glycogen mobilization. Its enzymatic activity degrades glycogen and produces glucose-1-phosphate, which is eventually transformed into trehalose or enters glycolysis. Present results demonstrate that in the fat body of *B. germanica* females, BgGlyP is activated in starvation conditions and that this activation is reduced in BgFoxO knockdown individuals (Fig. 3A), suggesting that BgFoxO is required for the activation of fat body GlyP expression. In parallel with BgGlyP mRNA reduction, a significant increase in fat body glycogen levels was observed in dsFoxO females (Fig. 3B), indicating that the degradation of glycogen is partially impaired in these animals. In contrast, a decrease in fat body glycogen levels was observed in dsFoxO fed animals (Fig. 3B). Provided that, in this case, no changes were observed in BgGlyP, a possible explanation would imply an effect of BgFoxO knockdown on glycogenesis.

In contrast to the results observed in fat body, no changes were shown in muscle BgGlyP mRNA levels due to dsFoxO treatment (Fig. 4B). Furthermore, no differences in glycogen content were observed between control and dsFoxO starved females (Fig. 4C). Nevertheless, as it occurred in fat body, a reduction in glycogen levels between control and dsFoxO was attained in fed females (Fig. 4C), which again points to an effect of BgFoxO knockdown on glycogenesis.

It has already been reported that AKH/HTH stimulates GlyP activity in the fat body via phosphorylation of the protein that converts the *b* form, which is active only in the presence of AMP, to the *a* form, which is active by itself [11]. Here, we have demonstrated that, besides this post-translational regulatory system, FoxO is required for activating GlyP expression in fat body of starved females. In the case of the muscle, we haven't found differences in BgGlyP mRNA which indicates that BgFoxO does not have the same effect in this tissue. In addition, extremely low AKH/HTH receptor levels have been reported in muscle compared to fat body in all the insects for which this has been studied [40-42]. All these results explain why we haven't found significant differences in muscle glycogen content between control and dsFoxO starved females.

Trehalose concentration in the haemolymph was lower in dsFoxO females for all the different experimental conditions (Fig. 5). A possible explanation of these lower trehalose levels would involve a higher level of trehalose entering into the fat body in dsFoxO animals for lipid synthesis. Higher levels of TAG in dsFoxO females (Fig. 2C) would point in this direction, although specific experiments should be performed to determine the trehalose uptake and lipogenesis rates.

Under starvation, glucose is made from glycerol, lactate and amino acids formed by proteolysis of muscle proteins [43]. In our case and with respect to the fat body enzymes related to gluconeogenesis, starvation induced a significant increase in BgPEPCK levels in control females, but this increase was abolished in the case of dsFoxO (Fig. 6A), which again indicates that BgFoxO induces BgPEPCK expression during starvation. Changes indicating the same conclusion were found in the case of BgAspAT, although the differences were not significant (Fig. 6B). Similar results showing FoxO-dependent activation of PEPCK expression have been reported in *D. melanogaster* [29, 44] and in mammals [24, 44]. And with respect to the regulatory effect of FoxO on AspAT expression, in *C. elegans* the presence of canonical binding sites for DAF-16 (the *C. elegans* FoxO homolog) in the promoter of AspAT has been demonstrated, and the specificity of DAF-16 binding was confirmed by chromatin immunoprecipitation (ChIP) [45].

The decrease in gluconeogenetic enzymes found in dsFoxO starved females will impair the use of gluconeogenic substrates for the production of glucose. One of these substrates may be glucogenic amino acids coming from the muscle. In our experiments a decrease in muscle protein was observed in the case of starved dsFoxO females (Fig. 6D). A possible explanation for these low protein levels is the low levels of glucose synthesis in the fat body, caused by the impaired gluconeogenesis in dsFoxO starved females, which would signal to the muscle to increase proteolysis.

In summary, our results indicate that FoxO induces the expression of genes related to catabolism in a situation of nutrient shortage in a basal insect, as is *B. germanica*. Of special relevance is the activation of the expression of the endocrine peptide HTH and the enzyme GlyP; because it connects the action of FoxO to an already known mechanism (the effect of HTH on GlyP protein activation) and because our result is analogous to the reported activity of FoxO in the activation of the transcription of mammalian hypothalamic peptides (Agouti-related peptide and neuropeptide Y) related to energy expenditure and which are important in different metabolic disorders [46].

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## FIGURE LEGENDS

**Fig. 1.** Hypertrehalosemic hormone (BgHTH) expression and effect of BgFoxO RNAi treatment on corpora cardiaca (CC) from *Blattella germanica* females. (A) BgHTH mRNA levels in CC from fed and starved 5 day-old adult females (n=5). (B and C) Effect of BgFoxO RNAi. dsRNA targeting BgFoxO (*dsFoxO*) or a non-homologous dsRNA (*dsMock*) were administrated in the first day of ootheca transport. The ootheca was removed 12 days later and a second gonadotrophic cycle was triggered. A group of females were normally fed, whereas a second group was maintained without food and only water was provided. Dissections were performed the day of the ootheca removal (0d) and 5 days later (5d Fed and 5d Starved). (B) BgFoxO mRNA levels (n=3-7). (C) BgHTH mRNA levels (n=3-7). Y-axis indicates copies per copy of BgActin 5C. Results are expressed as the mean  $\pm$  S.E. Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.005).

**Fig. 2.** Effect of BgFoxO RNAi treatment on fat body from *Blattella germanica* females. Experimental procedure was the same as in Fig. 1. (A) BgFoxO mRNA levels (n=6-16). (B) Brummer lipase (BgBmm) mRNA levels (n=5-7). (C) Triacylglycerides (TAG) levels (n=5-6). (D) Representative images of fat bodies from dsMock and dsFoxO 5d fed females stained with Nile Red. Scale bars: 50  $\mu$ m. (A) and (B) Y-axis indicate copies per copy of BgActin 5C. Results are expressed as the mean  $\pm$  S.E. Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05; \*\**P*<0.005; *P*<0.0001).

**Fig. 3.** Effect of BgFoxO RNAi treatment on fat body from *Blattella germanica* females. Experimental procedure was the same as in Fig. 1. (A) Glycogen phosphorylase (BgGlyP) mRNA levels (n=7-9). (B) Glycogen levels (n=5-6). (A) Y-axis indicates copies per copy of BgActin 5C. Results are expressed as the mean  $\pm$  S.E. Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05; \*\**P*<0.005; *P*<0.0001).

**Fig. 4.** Effect of BgFoxO RNAi treatment on leg muscle from *Blattella germanica* females. Experimental procedure was the same as in Fig.1. (A) BgFoxO mRNA levels (n=6-11). (B) Glycogen phosphorylase (BgGlyP) mRNA levels (n=5-10). (C) Glycogen levels (n=4-6). (A) and (B) Y-axis indicate copies per copy of BgActin 5C. Results are expressed as the mean  $\pm$  S.E. Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05; \*\**P*<0.0005; \*\*\**P*<0.0001).

**Fig. 5.** Effect of BgFoxO RNAi treatment on haemolymph trehalose levels of *Blattella germanica* females. Experimental procedure was the same as in Fig. 1. Results are expressed as the mean  $\pm$  S.E, (n=5-10). Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05).

**Fig. 6.** Effect of BgFoxO RNAi treatment on fat body and leg muscle from *Blattella germanica* females. Experimental procedure was the same as in Fig. 1. (A) Fat body phosphoenolpyruvate carboxylase (BgPEPCK) mRNA levels (n=6-9). (B) Fat body aspartate aminotransferase (BgAspAT) mRNA levels (n=5-10). (C) Fat body pyruvate carboxylase (BgPC) mRNA levels (n=5-10). (D) Leg protein levels (n=5-6). (A), (B) and (C) Y-axis indicate copies per copy of BgActin 5C. Results are expressed as the mean  $\pm$  S.E. Asterisks represent significant differences between dsMock and dsFoxO (Student's *t* test, \**P*<0.05; \*\**P*<0.001).

Figure 1

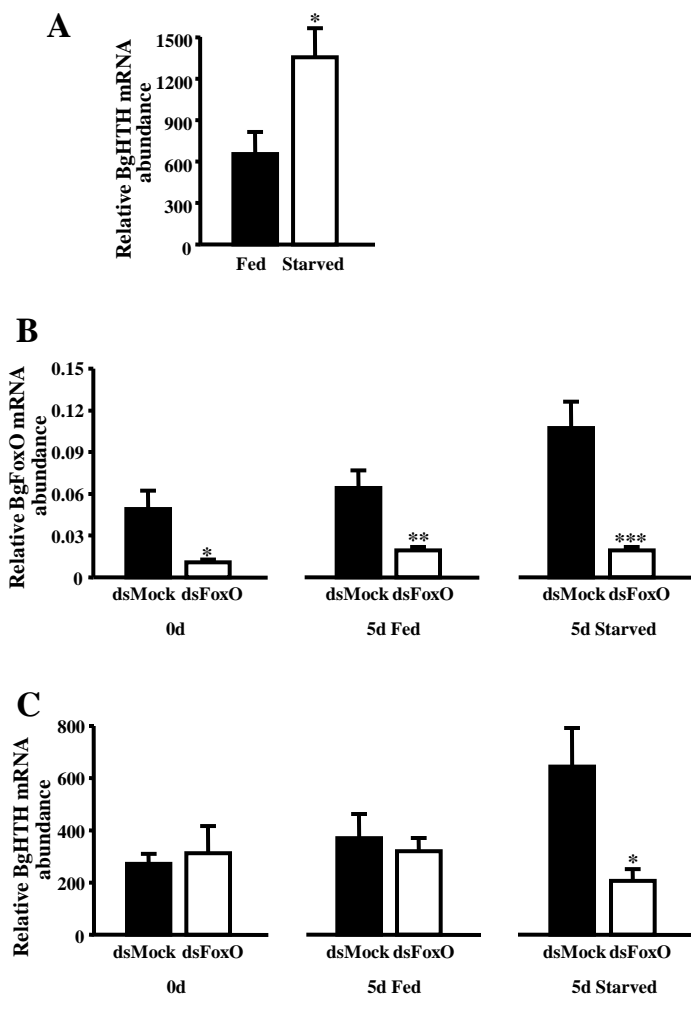


Figure 2

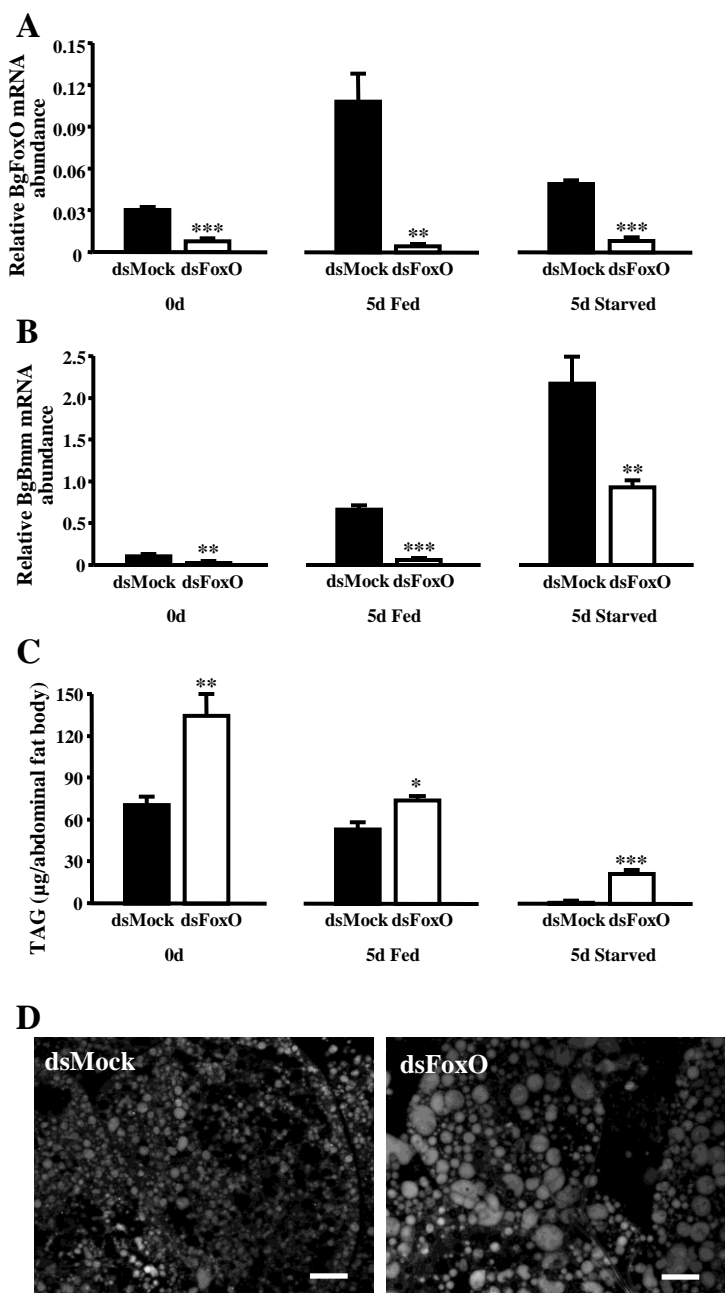


Figure 3

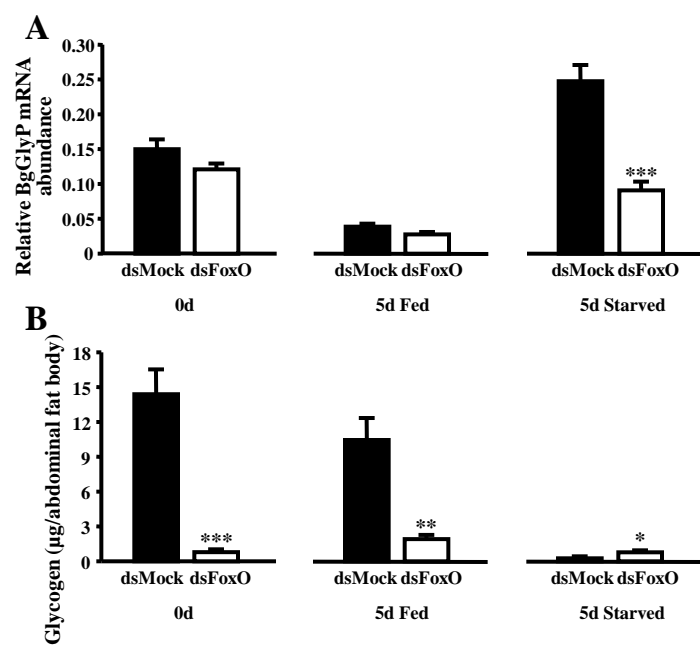


Figure 4

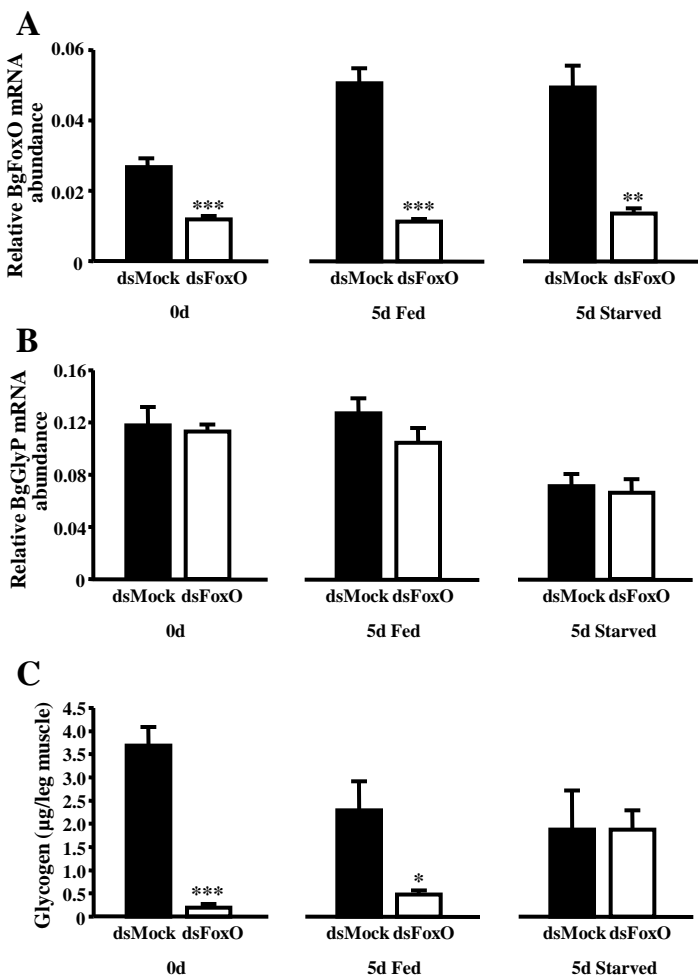




Figure 5

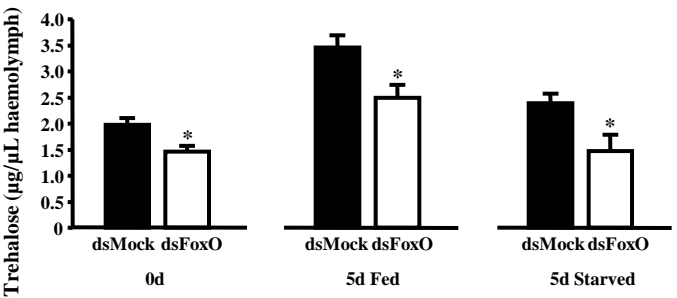
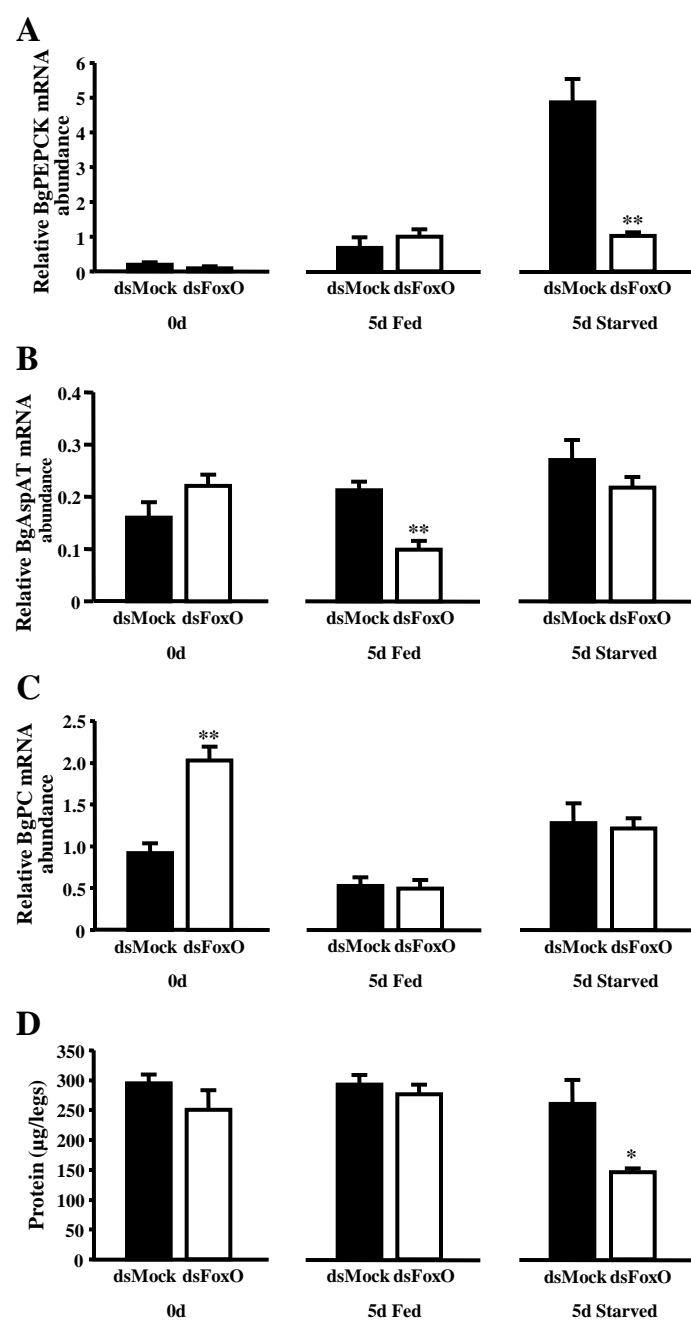


Figure 6



**Table 1.** Primer sequences.

**Primers used for cloning**

BgBmm F1	TTYGCN GGNTGYGGNTT
BgBmm F2	TAYGAYGGNAARAAYGTNAT
BgBmm R1	GTNACRTCRAADATYTCNGT
BgBmm R2	GCRTCRTCRAANCCYTG
BgGlyP F1	AARTTYCARAAYAARACNAAYGG
BgGlyP F2	ATGATHGGNGGNAARGCNGC
BgGlyP R1	CATYTCNACRTTNGCNCCRTC
BgAspAT F1	TGYGCNCAYAAAYCCNACNGG
BgAspAT F2	TTYGAYWSNGCNTAYCARGG
BgAspAT F3	TTTGGTGCAGGTGATGTTGCAAGA
BgAspAT R1	TANSWRAACATNCCDATYTG
BgPC F1	CAYGARTGYCCNTGGGARMG
BgPC F2	CCNAAYATHCCNTTYCARATG
BgPC R1	TTYTGNACCATRAAYTGNGC
BgPC R2	TTYTTNACRTCYTCRAARAA
BgPEPCK F1	GGNTGGYTNGCNGARCAYATG
BgPEPCK R1	GGNCKCATNGCRAANGGRTC
BgPEPCK R2	TCRAAYTTCATCCANGCDATRTC

**Primers used for qPCR**

BgHth qPCR Fwd	AGACCTTGGTAGTTGTGGTGGC
BgHth qPCR Rv	GGATTCCTGATCTCTTGCCTGT
BgBmm qPCR Fwd	GACATCTGTCCCCGTGATTG
BgBmm qPCR Rv	CCTGTAGATATTGTGGCGAGACAA
BgGlyP qPCR Fwd	TCATGCCTGCGACAGATCTC
BgGlyP qPCR Rv	CTCCATTCAACTGGAAC TTCATGT
BgAspAT qPCR Fwd	CAAGGATGATGCTCAAAGAACG
BgAspAT qPCR Rv	AACAATGCGAGCTCCATGG
BgPC qPCR Fwd	CGTTGCATCTCTTCAAGGCA
BgPC qPCR Rv	TGTGCGTGTTTGTTC CCAAT
BgPEPCK qPCR Fwd	ATCTTGCCATGATGTGCCCT
BgPEPCK qPCR Rv	TTATTTGATGTGCCTGGTGCA